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Arabidopsis PASTICCINO2 Is an Antiphosphatase Involved in Regulation of Cyclin-Dependent Kinase A[W]

Marco Da Costa,[a,1] Liên Bach,[a] Isabelle Landrieu,[b] Yannick Bellec,[a] Olivier Catrice,[c] Spencer Brown,[c] Lieven De Veylder,[d] Guy Lippens,[b] Dirk Inzé,[d] and Jean-Denis Faure[a,2]

[a] Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, F-78026 Versailles Cedex, France
[b] Institut Pasteur de Lille, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8525, Université de Lille, F-59019 Lille Cedex, France
[c] Dynamique de la Compartimentation Cellulaire, Institut des Sciences du Végétal, Unité Propre de Recherche 2355, Centre National de la Recherche Scientifique, F-91198 Gif-sur-Yvette, France
[d] Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, Ghent University, B-9052 Ghent, Belgium

***PASTICCINO2* (*PAS2*), a member of the protein Tyr phosphatase-like family, is conserved among all eukaryotes and is characterized by a mutated catalytic site. The cellular functions of the Tyr phosphatase-like proteins are still unknown, even if they are essential in yeast and mammals. Here, we demonstrate that PAS2 interacts with a cyclin-dependent kinase (CDK) that is phosphorylated on Tyr and not with its unphosphorylated isoform. Phosphorylation of the conserved regulatory Tyr-15 is involved in the binding of CDK to PAS2. Loss of the PAS2 function dephosphorylated *Arabidopsis thaliana* CDKA;1 and upregulated its kinase activity. In accordance with its role as a negative regulator of the cell cycle, overexpression of *PAS2* slowed down cell division in suspension cell cultures at the G2-to-M transition and early mitosis and inhibited *Arabidopsis* seedling growth. The latter was accompanied by altered leaf development and accelerated cotyledon senescence. PAS2 was localized in the cytoplasm of dividing cells but moved into the nucleus upon cell differentiation, suggesting that the balance between cell division and differentiation is regulated through the interaction between CDKA;1 and the antiphosphatase PAS2.**

INTRODUCTION

In multicellular organisms, cell division and cell differentiation have to be coordinated during development. This statement is especially true for plants that carry on continuous organogenesis in the meristems, where cells have to maintain their proliferative potential as well as initiate differentiation to produce new organs. Cell cycle regulators are required for the control of cell cycle transitions but seem also to be involved in coordinating transitions between cell proliferation and cell differentiation (Gutierrez, 2005).

In eukaryotes, cell division is regulated by phosphorylation events performed by cyclin-dependent kinases (CDKs) (Inzé, 2005). The cell cycle machinery is conserved among eukaryotes, and, in particular, several CDKs have been characterized in plants (Vandepoele et al., 2002). CDKA has been identified as the bona fide CDK because of the presence of the conserved PSTAIRE motif and by its ability to complement the yeast *Schizosaccharomyces pombe cdc2* mutant (Colasanti et al., 1991; Ferreira et al., 1991). Of the plant-specific CDKs with the PPTALRE and PPTTLRE motifs, CDKB can not complement the *S. pombe cdc2* mutant but is nonetheless involved in the G2-to-M transition (Porceddu et al., 2001; Boudolf et al., 2004). Similarly to CDKs, a large family of cyclins has been described in several plant species (Inzé, 2005). Correct cell cycle progression requires a tight control of protein and activity levels of CDK/cyclin complexes (De Veylder et al., 2003; Dewitte and Murray, 2003). For instance, transcriptional regulation of the expression of cyclins is important for the G1-to-S transition, but posttranslational modifications that affect CDK complex stability and activity are essential for many cell cycle steps (Inzé, 2005). Irreversible inactivation of the CDK/cyclin complex results from ubiquitin-mediated degradation of the cyclins (Genschik et al., 1998; Koepp et al., 1999). One of the most important posttranslational modifications of CDKs involves protein phosphorylation. CDKs are initially activated by cyclin association and by phosphorylation on a Thr residue in the conserved T-loop (Connell-Crowley et al., 1993; Solomon, 1993). CDK/cyclin complexes are reversibly inactivated by phosphorylation of Thr-14 and Tyr-15 of CDK (Morgan, 1995). Phosphorylation is mediated by the WEE1/MYK1/MYT protein kinase, while dephosphorylation is caused by the dual specificity protein Tyr phosphatase (dsPTP) CDC25.

[1] Current address: Cell Proliferation Group, Medical Research Council Clinical Sciences Centre, Imperial College Faculty of Medicine, Hammersmith Hospital Campus, Du Cane Road, London, W12 0NN, UK.
[2] To whom correspondence should be addressed. E-mail jean-denis.faure@versailles.inra.fr; fax 33-130-83-3099.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jean-Denis Faure (jean-denis.faure@versailles.inra.fr).
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Dephosphorylation of CDK by CDC25 at the G2-to-M transition is a rate-limiting step for CDK activation and, thus, for cell cycle progression (Donzelli and Draetta, 2003).

The plant WEE1 regulatory kinase was identified in *Arabidopsis thaliana* and maize (*Zea mays*) (Sun et al., 1999; Sorrell et al., 2002). Until recently, no gene with significant primary sequence similarity to CDC25 could be detected in the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), although the CDC25-like activity was found to be involved in the G2-to-M transition of *Nicotiana plumbaginifolia* cells (Zhang et al., 1996, 2005). Accordingly, an *Arabidopsis* CDC25 isoform with a tertiary structure similar to that of the human CDC25 and able to activate in vitro the CDK/cyclin complex has only recently been cloned (Landrieu et al., 2004a, 2004b).

In animals, tumorigenesis is often preceded by the deregulation of cell cycle components that triggers ectopic cell proliferation. Surprisingly, plants seem to be strikingly resistant to unrestricted growth similar to mammalian hyperplasia or cancer when cell cycle genes are deregulated (Cockcroft et al., 2000; Gutierrez, 2005). Nonetheless, direct genetic screens for ectopic cell proliferation and tumor-like development have identified several mutants in *Arabidopsis* (Faure et al., 1998; Bouton et al., 2002; Frank et al., 2002), among which the *pasticcino* (*pas*) mutants (*pas1*, *pas2*, and *pas3*) have been characterized by ectopic cell proliferation in the apical part, which is a cytokinin-enhanced process (Faure et al., 1998). These *pas* mutants have impaired embryo and seedling development associated with modified cytokinin and auxin sensitivity (Harrar et al., 2003). The three *PAS* genes have been identified as members of a conserved gene family in eukaryotes (Vittorioso et al., 1998; Bellec et al., 2002; Baud et al., 2004). *PAS2* encodes the *Arabidopsis* member of a new PTP family, the PTP-LIKE (PTPL) family, originally described in mice and humans and characterized by mutations in the active site that conferred phosphatase inactivity (Uwanogho et al., 1999; Bellec et al., 2002; Pelé et al., 2005). The cellular function of this protein family is still unknown but seems to be critical because the absence of the *PAS2* homolog in yeast is lethal and in mammals leads to severe defects in myofibril differentiation (Bellec et al., 2002; Pelé et al., 2005). The discovery of a putative inactive PTP involved in plant cell proliferation raised the possibility that the plant cell cycle might be regulated by an antiphosphatase.

Here, we show that PAS2 is indeed a component of the CDKA;1/cyclin complex by its interaction with phosphorylated CDKA;1. PAS2 protects the phosphorylated CDKA;1 residues from phosphatase activities and thus regulates CDKA;1 phosphorylation status and kinase activity; however, its absence causes CDKA;1 dephosphorylation and increases cell competence to divide. A mechanism is proposed based on the competitive action of an antiphosphatase and a dsPTP on CDKA;1 to control cell competence for division.

RESULTS

PAS2 Interacts with CDKA;1 in Vivo

The sequence similarity between PAS2 and members of the PTPL family suggested that PAS2 could be involved in protein phosphorylation. The fact that PTPL members are characterized by a mutated catalytic site of PTP prompted us to consider PAS2 as a competitor of an active PTP for a phosphorylated substrate. *Arabidopsis* CDKA;1 appeared to be a potential target of PAS2 for several reasons. First, this main regulator of the cell cycle is one of the few plant proteins in which a regulatory Tyr phosphorylation site is conserved. Then, CDKA dephosphorylation by a CDC25-like activity is required for cell cycle progression of *N. plumbaginifolia* (Zhang et al., 2005). In particular, a CDC25-like activity has been found to be necessary for cytokinin regulation of the G2-to-M transition of the cell cycle. Finally, the *pas2* mutants show cell dedifferentiation and proliferation, which are specifically enhanced by cytokinins, suggesting that both PAS2 and cytokinins could regulate CDKA;1 phosphorylation by targeting CDC25 activity.

We tested whether PAS2 and CDKA;1 interact in vivo by purifying PAS2-interacting proteins by pull-down assays (Figure 1A). Total proteins from *Arabidopsis* cell cultures (lane 1) were loaded on a column with PAS2 (lane 2) or on an empty control column (lane 3). The analysis of PAS2 binding proteins by protein gel blots showed that CDKA;1 was specifically retained on the PAS2 column (Figure 1B). The reverse experiment was performed by affinity purification of the active CDKA;1 complexes from *Arabidopsis* cell cultures with $p10^{CKS1At}$ columns (Figure 1C, bottom). Protein gel blot analysis with anti-PAS2 serum indicated that PAS2 copurified with CDKA;1 (Figure 1C, top).

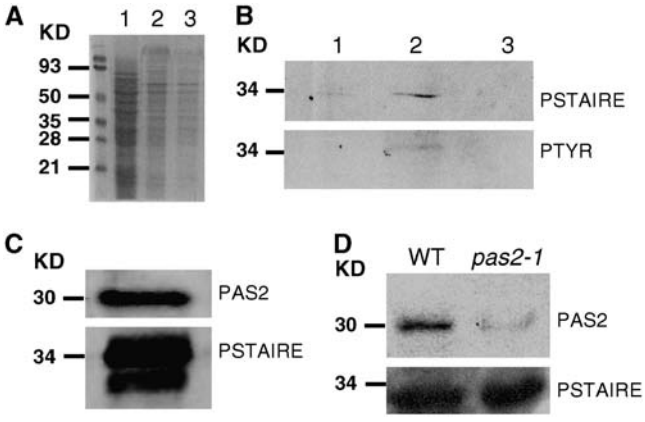

Figure 1. Interaction of PAS2 with CDKA;1 in Vivo.

(A) and **(B)** Purification of CDKA;1 by recombinant PAS2 (PAS2:His) from total protein extracts of *Arabidopsis* cells. The pull-down assay was performed with a PAS2:His column. Proteins were detected by Coomassie staining **(A)** and by protein gel blots **(B)** from total protein extract of *Arabidopsis* cells (1), protein extract after purification on PAS2:His column (2), and protein extract after purification on empty column (3). Protein gel blot analysis was performed with anti-PSTAIRE (PSTAIRE) and antiphosphotyrosine (PTYR).

(C) Copurification of PAS2 with the CDK complex. Detection of PAS2 (anti-PAS2; PAS2) and CDKA;1 (PSTAIRE) after CDK purification by $p10^{CKS1At}$ beads from protein extracts of *Arabidopsis* cells.

(D) Association of PAS2 with the CDK complex in *Arabidopsis* seedlings. Detection of PAS2 and CDKA;1 (PSTAIRE) after CDK purification by $p10^{CKS1At}$ beads from protein extracts of wild-type plants or *pas2-1* mutant plants.

A similar experiment was performed with wild-type *Arabidopsis* seedlings, and, as expected, PAS2 was also associated with CDKA;1 (Figure 1D). As anticipated, a low amount of PAS2 was found in the complex with CDKA;1 in a weak *pas2* allele, which was characterized by a strongly reduced steady state level of the PAS2 mRNA (Bellec et al., 2002). Altogether, these data demonstrate that PAS2 interacts with the CDKA;1 protein complex in cell cultures as well as in seedlings.

PAS2 Interacts Only with Phosphorylated CDK and Inhibits CDKA;1 Dephosphorylation

The PTP motif in the PAS2 sequence suggested that protein phosphorylation was involved in the PAS2 function. The recombinant PAS2 had no phosphatase activity (Bellec et al., 2002), but the protein could have still retained its ability to bind a phosphorylated substrate. We tested this hypothesis using an in vitro binding assay with the recombinant PAS2:His and CDKA;1:maltose binding protein (MBP). The recombinant CDKA;1:MBP was purified from *Escherichia coli* and phosphorylated by Src Tyr kinase in vitro. Phosphorylation was monitored by radioactive incorporation of γ^{32}P-ATP (data not shown) and by protein gel blots with an antiphosphotyrosine antibody (Figure 2A). We verified that Tyr-15, which is an important regulatory residue of CDKA;1 and a substrate of CDC25, was phosphorylated by Src Tyr kinase. Both the unphosphorylated and phosphorylated recombinant CDKA;1:MBP proteins were loaded onto a PAS2 column, and bound proteins were eluted and analyzed by protein gel blots (Figure 2B). Only the phosphorylated CDKA;1:MBP was retained on the PAS2 column, demonstrating that the PAS2-CDKA;1 interaction requires CDKA;1 phosphorylation on Tyr residues. Accordingly, a Tyr phosphorylated protein of a size similar to that of CDKA;1 was also affinity purified by PAS2 from *Arabidopsis* cell culture extract (Figure 1B).

We then investigated the role of Tyr-15 phosphorylation in PAS2 binding to CDK using the specific kinase WEE1. Because the phosphorylation specificity of the *Arabidopsis* WEE1 had never clearly been addressed, we used the well-characterized human WEE1 kinase that phosphorylated specifically Tyr-15 of CDK2 (Morgan, 1997). Human CDK2 and *Arabidopsis* CDKA;1 share 69% identity at the protein level and a complete identity over 17 residues around Tyr-15. The glutathione *S*-transferase

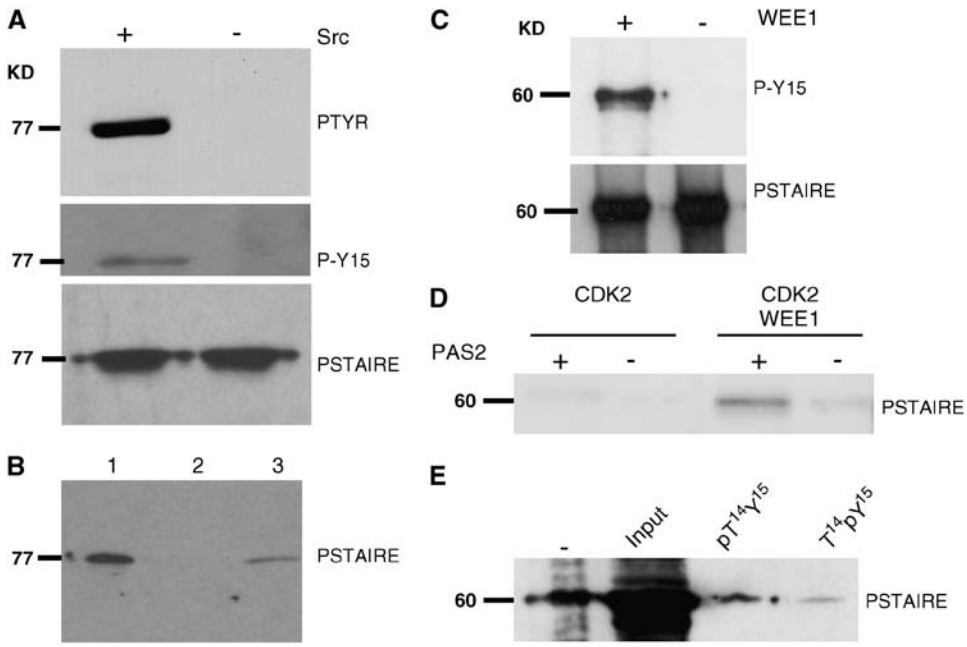

Figure 2. Interaction of PAS2 with Phosphorylated CDKA;1.

(A) In vitro phosphorylation of CDKA;1:MBP. CDKA;1:MBP was incubated (+) or not (−) with Src kinase and analyzed by protein gel blotting with antiphosphotyrosine (PTYR), antiphosphotyrosine 15 (P-Y15), and anti-PSTAIRE antibodies.

(B) Tyr phosphorylation of CDKA;1 is required for the interaction with PAS2. Incubation of CDKA;1:MBP phosphorylated or not by Src kinase with PAS2:His column. Interaction between PAS2 and CDKA;1:MBP or CDKA;1:MBP Y-P was monitored by protein gel blots with the anti-PSTAIRE antibody. (1) CDKA;1:MBP, (2) elution of CDKA;1:MBP from PAS2:His column, and (3) elution of CDKA;1:MBP Y-P from PAS2:His column.

(C) In vitro phosphorylation of GST:CDK2 by WEE1. GST:CDK2 was coproduced in *E. coli* with (+) or without (−) the WEE1 kinase and analyzed by protein gel blotting with antiphosphotyrosine 15 (P-Y15) and anti-PSTAIRE antibodies.

(D) WEE1 phosphorylation of CDK2 is required for the interaction with PAS2. The GST:CDK2 fusion protein phosphorylated or not by WEE1 kinase was incubated with PAS2:His column, and the interaction was monitored by protein gel blots with the anti-PSTAIRE antibody.

(E) The interaction of PAS2 with phosphorylated CDK2 is competed off with pTyr-15 peptide. The experiment was similar to **(D)** except that the conserved peptide EKVEKIGEGTYGVVYK phosphorylated on Thr (pT^{14}) or phosphorylated on Tyr (pT^{15}) was included or not (−) in the binding experiment, and the interaction was monitored by protein gel blots with the anti-PSTAIRE antibody.

(GST):CDK2 fusion protein was produced in *E. coli* in the absence or in the presence of WEE1 as previously described (Welburn and Endicott, 2004). The fusion proteins were purified, and Tyr-15 was found to be phosphorylated only in the presence of WEE1 (Figure 2C). Both fusion proteins were then incubated with a PAS2:His column and an empty column. Only the WEE1-treated GST:CDK2 fusion protein interacted with PAS2, demonstrating that phosphorylation of the Tyr-15 residue was necessary for PAS2 binding. Finally, to confirm the involvement of pTyr-15, we performed competition experiments with the conserved peptide EKVEKIGEGTYGVVYK overlapping Thr-14–Tyr-15. Peptides phosphorylated on Tyr-15 but also on Thr-14 were used to displace WEE1-phosphorylated GST:CDK2 binding to PAS2. As shown in Figure 2E, both pTyr-15 and pThr-14 peptides competed off GST:CDK2 binding to PAS2 even if phosphorylation of Tyr-15 seemed more efficient in the competition experiment. Altogether, our results showed that PAS2 binds only phosphorylated CDK and that the conserved Tyr-15 and Thr-14 are involved in the interaction.

We checked whether PAS2 binding could protect CDKA;1 from dephosphorylation. According to this hypothesis, loss of PAS2 should improve the access of phosphatases to CDKA;1 and, in turn, decrease CDKA;1 Tyr phosphorylation. The CDKA;1 complex was purified from the wild type and the *pas2-1* mutant, and the CDKA;1 phosphorylation status and kinase activity were measured. Compared with the wild type, the CDKA;1 Tyr phosphorylation level in the *pas2-1* mutant had decreased severely, and, accordingly, the associated CDKA;1 kinase activity had increased (Figures 3A and 3B). In conclusion, PAS2 maintains CDKA;1 in an inactive state by protecting CDKA;1 from dephosphorylation by phosphatases.

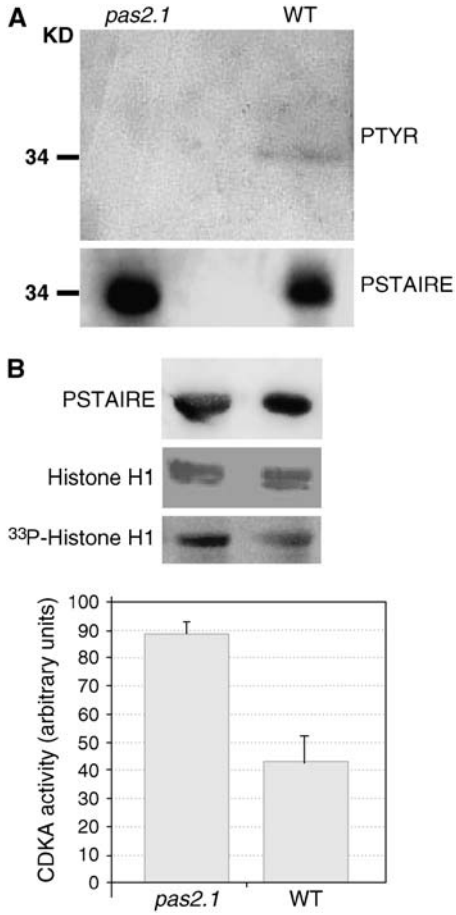

Figure 3. Inhibition of CDKA;1 Phosphorylation and Activity by PAS2.

(A) Decreased Tyr phosphorylation in *pas2-1* mutant by CDKA;1. CDK complexes were purified on $p10^{CKS1At}$ from wild-type and *pas2-1* mutant plants. Phosphorylated CDKA;1 was revealed by anti-PTYR and anti-PSTAIRE protein gel blots.

(B) Increase of the CDK-associated kinase activity in *pas2-1* mutant plants. The *Arabidopsis* CDK complexes were purified on $p10^{CKS1At}$, and the kinase activity was quantified by monitoring the histone H1 phosphorylation. Data were normalized according to the CDKA;1 protein level as quantified by the protein gel blots with anti-PSTAIRE antibody. The kinase activity represents the result of three independent experiments. Error bars represent SE.

PAS2 Overexpression Alters Cell Division in Tobacco Cells

Loss-of-function of *PAS2* leads to ectopic cell proliferation. This feature is consistent with its role as a CDKA;1 inhibitor. By contrast, an increase in the PAS2 protein in the cell should alter cell division and, eventually, impair development. To assess whether PAS2 is involved in the regulation of cell division, we overexpressed *PAS2* in tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) suspension cells. Because the PAS2 effect could be rapidly counterselected after several rounds of subcultures, we expressed *PAS2* under the control of an inducible Cre/Lox system (Joubès et al., 2004). Briefly, in this system, the *PAS2* gene is cloned under the control of the constitutive cauliflower mosaic virus 35S promoter, but a green fluorescent protein (GFP) spacer with a transcription termination site prevents its expression. The GFP spacer is flanked with the Lox recombination sites and can be excised in the presence of the CRE recombinase. A ligand-dependent CRE-GR construct expressed under a heat shock promoter was used to tightly control the recombinase activity. Thus, GFP excision and *PAS2* expression are induced by the expression of the construct *pHSP-CRE:GR* after heat shock (HS) and dexamethasone (Dex) treatment (Figures 4A and 4B). The HS/Dex treatment did not affect the BY-2 cell cultures (Figure 4C; Joubès et al., 2004). Without HS/Dex treatment, 90 to 95% of the BY-2 cells harboring the *35S-lox:GFP:lox:PAS2* construct ($PAS2^{loxGFPlox}$) were GFP positive, and, correspondingly, only a low basal level of *PAS2* expression was detected in the culture (Figures 4A and 4B). By contrast, upon HS/Dex treatment, >80% of the cells became GFP negative after 3 d and accumulated *PAS2* transcripts as early as 7 h after treatment (Figures 4A and 4B). The effect of the *PAS2* expression could be observed 24 h after induction by a decrease in cell culture growth (Figure 4C). To check whether the effect of *PAS2* on culture growth was related to cell division, we analyzed the cell cycle progression in synchronized $PAS2^{loxGFPlox}$ cells. BY-2 cells were first treated with HS/Dex and 24 h later blocked at the G1-to-S transition by aphidicolin treatment. Aphidicolin reversibly arrests cells in early S phase by inhibiting DNA polymerase α and δ (Sala et al., 1983; Nagata et al., 1992). Cells were released from the

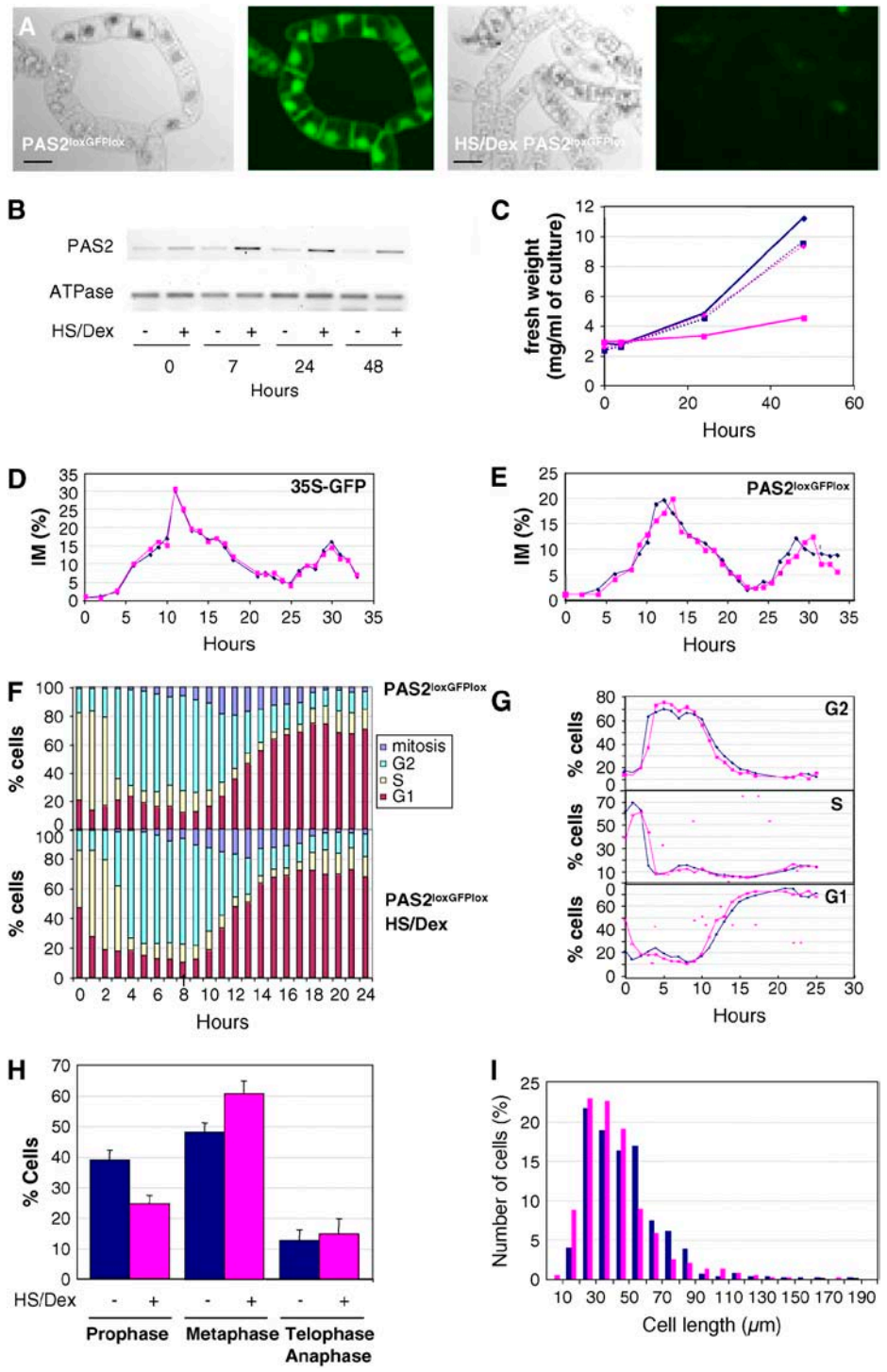

Figure 4. Inducible *PAS2* Overexpression in BY-2 Cells.

(A) Extensive GFP activity in BY-2 cells transformed with PAS2^{loxGFPlox} (left). Three days after HS and Dex induction, no GFP fluorescence could be detected (right). For each condition, BY-2 cells were analyzed in light transmission and for GFP fluorescence. Bars = 30 μm.

(B) Increase in *PAS2* expression after HS/Dex treatment. Time course of *PAS2* expression was measured by PCR on reverse transcription of RNA from PAS2^{loxGFPlox} BY-2 cells induced (+) or not (−).

(C) Growth inhibition of induced (pink) versus uninduced (blue) PAS2^{loxGFPlox} cell cultures. HS/Dex treatment did not affect growth of the control GFP cell culture (blue dashed line) compared with the untreated condition (pink dashed line).

aphidicolin block, and cell cycle progression was monitored by measuring the mitotic index. The aphidicolin block associated with HS and Dex did not modify cell cycle progression because control 35S-GFP transgenic BY-2 cells entered mitosis at the same time whether they had been treated with HS/Dex or not (Figure 4D). Untreated PAS2^{loxGFPlox} showed, like the control cell lines, a peak of mitosis at 12 h and then at 28 h after aphidicolin release. By contrast, HS/Dex-treated PAS2^{loxGFPlox} cells reached mitosis 13 and 30 h after aphidicolin release, corresponding to a 1- and 2-h delay compared with untreated cells, respectively (Figure 4E). This delay in mitosis was not caused by a stress response from the combination of HS and aphidicolin treatment because a similar delay was observed in the second mitosis. The lag in mitosis entry of PAS2^{loxGFPlox}-treated cells was on average 1.6 h $\pm$ 0.9 h for five independent experiments, while control cells treated or not always entered into mitosis simultaneously. The fact that entry into mitosis had already been delayed during the first cycle after aphidicolin block suggested that PAS2 acts in S or G2 phases or at the transition between G2 and mitosis. To discriminate between these hypotheses, the cell cycle phases were measured by flow cytometry for the first 25 h of the synchronization experiment (Figure 4F). Treated PAS2^{loxGFPlox} cells were arrested earlier in S phase than untreated cells after aphidicolin block, but time of entry into G2 phase was similar for both cell lines (Figure 4G, top and middle). Thus, the delay observed in S phase could not explain the difference in mitosis timing between treated and untreated cell cultures. While both types of cells entered the G2 phase synchronously, HS/Dex-treated cells had a shorter G2 phase because cells exited G2 1 h earlier than control cells (Figure 4G, top). The same pattern of early entry in G2 of HS/Dex-treated PAS2^{loxGFPlox} cells was observed in two independent experiments. The delay in mitosis of the HS/Dex cell population must then originate from an inhibition of the cell progression through mitosis. Thus, we checked whether *PAS2* expression had an effect on specific mitotic phases by scoring the frequency of the different mitotic figures. Treated cells had an unbalanced prophase:metaphase ratio (Figure 4H), suggesting that the *PAS2* overexpression inhibited early mitosis. While the cell cycle study on BY-2 cultures demonstrated that PAS2 has a direct negative effect on cell division, this effect is probably not the only cause of growth inhibition. The reduction of PAS2^{loxGFPlox} cell culture growth rate after induction could also result from an inhibition of cell expansion. The measurements of PAS2^{loxGFPlox} cell length 48 h after HS/Dex induction showed that PAS2 induction led to a shift of the cell length distribution toward smaller cells (Figure 4I). In summary, the induction of PAS2 in BY-2 cells reduced the growth of cell culture, which was caused by a combined inhibition of cell cycle progression and cell expansion.

PAS2 Overexpression Alters Plant Growth

The effect of *PAS2* overexpression in *Arabidopsis* was analyzed with transgenic plants overexpressing *PAS2* or *PAS2:GFP* fusions. All the constructs were functional because they could complement the *pas2-1* mutant (see Supplemental Figure 1 online). High GFP-producing plants were retained for further analysis because PAS2 accumulation could be monitored easily in the whole seedlings throughout development (Figures 5A and 5B). *PAS2* expression relative to *EF1*α was quantified by RT-PCR in *35S-PAS2:GFP* seedlings compared with the wild type (Columbia-0) (Figure 5C). The transgenic plants accumulated 30-fold more *PAS2* transcripts than untransformed wild type. Several independent transgenic lines with high GFP staining had obvious developmental defects with slowed growth and stunted phenotype (Figure 5A). Cotyledons were smaller in *35S-PAS2:GFP* seedlings compared with those in the wild type and seemed to have an accelerated senescence. The development of first leaves was often altered: either the first leaves were absent or had a delayed growth with primordia-like shapes or reduced leaf blades (Figure 5D). Epidermal cells of young cotyledons of *35S-PAS2:GFP* seedlings were similar to those of the wild type, suggesting that the smaller size of the cotyledons was caused by a reduced number of cells (Figure 5E).

PAS2 Subcellular Distribution

The intracellular distribution of PAS2 protein was investigated in the root cells of transgenic *Arabidopsis* expressing the construct *35S-PAS2:GFP* (Figures 5F and 5G). PAS2:GFP was excluded from the nucleus in the root meristem (Figure 5F) and in the elongation zone as well (data not shown). On the contrary, most of the cells in the differentiated zone displayed cytosolic but also nuclear distribution of the PAS2:GFP protein fusion (Figure 5G). The presence of PAS2 in the nucleus could also be seen in root hairs (see Supplemental Figure 2 online).

Figure 4. (continued).

(D) and **(E)** Delay in cell cycle progression induced by PAS2. HS/Dex treatments did not modify cell cycle progression. Mitotic index was measured, after release from aphidicolin block, in *35S-GFP* and *PAS2^{loxGFPlox}* cell cultures that were induced (pink line) or not (blue line). While HS/Dex treatment did not affect growth of the control GFP cell culture **(D)**, it delayed PAS2^{loxGFPlox} cell mitosis **(E)**.

(F) Cytometric analysis of the cell cycle of synchronized cells uninduced (top) and induced (bottom) from the experiment presented in **(E)**.

(G) Time course progression through the cell cycle of uninduced (blue) and induced (pink) PAS2^{loxGFPlox} cells in G2 (top panel), S (middle panel), and G1 phases (bottom panel), respectively.

(H) Distribution of prophase, metaphase, and anaphase plus telophase during mitosis of uninduced (blue) and induced (pink) PAS2^{loxGFPlox} cells. Mitotic figures were sampled in five time points during the first peak of mitosis of synchronized cells described in **(E)**. Approximately 200 mitotic figures were counted for each time point. Error bars represent SE.

(I) Cell length distribution of uninduced (blue) and induced (pink) PAS2^{loxGFPlox} cells. Cell length was measured 48 h after induction on 439 uninduced and 575 induced cells.

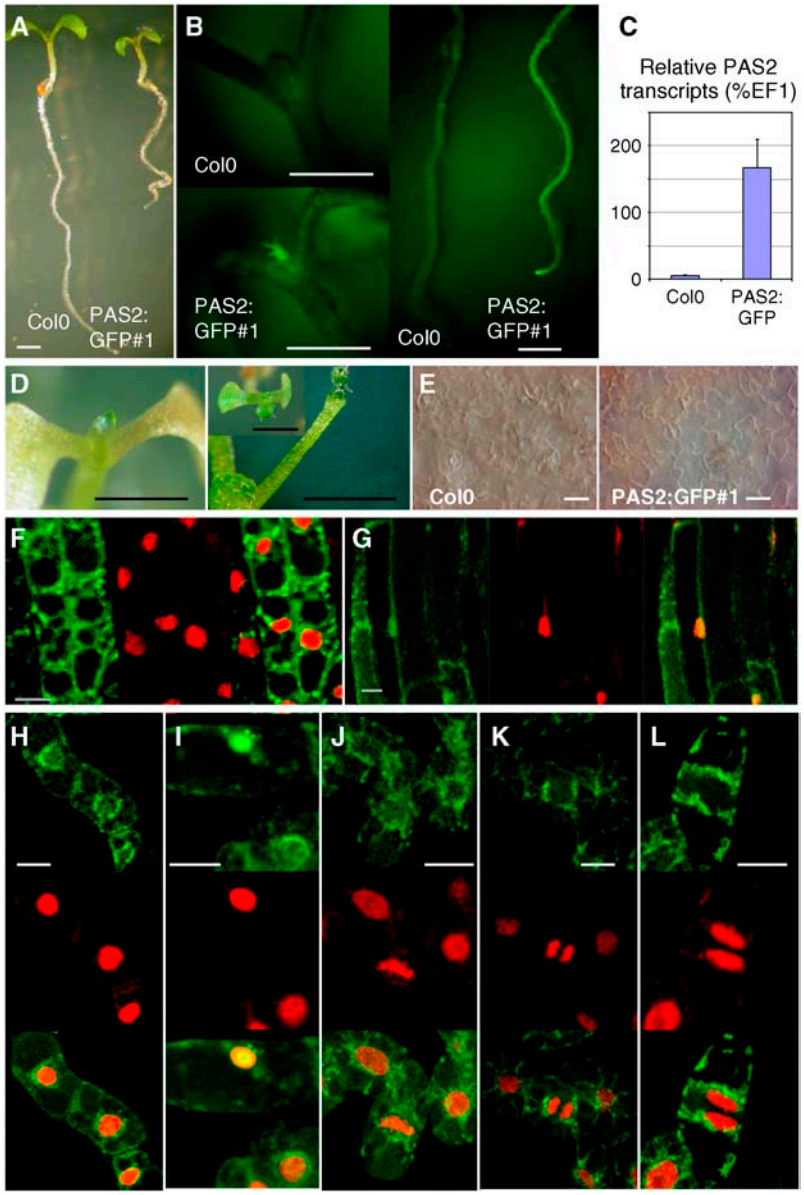

Figure 5. *PAS2:GFP* Expression in *Arabidopsis*.

(A) Growth inhibition by stable expression of 35S-PAS2:GFP in 10-d-old seedlings.
(B) GFP activity in seedling meristem and primary root of transgenic seedlings compared with control (Columbia-0).
(C) Expression level of PAS2 in 35S-PAS2:GFP seedlings compared with the wild type. Real time RT-PCR amplification of PAS2 transcript was normalized against EF1α. Error bars represent SE.
(D) Altered development of young leaves in 10-d-old transgenic *35S-PAS2:GFP* seedlings with fused (left), finger-shaped (right), or missing leaf primordia (inset).
(E) Cotyledon epidermis cells of *35S-PAS2:GFP* plants compared with the wild type.
(F) and **(G)** Subcellular distribution of PAS2:GFP changed according to cell type. In root meristem with actively dividing cells, PAS2:GFP fusion is mainly present in the cytosol **(F)**. In differentiated cells in the root hair initiation zone, PAS2:GFP shows cytosolic and nuclear localization **(G)**. For each panel, the GFP signal, the DRAQ5 nuclear staining, and merged image are shown in the left, middle, and right panels, respectively.
(H) to **(L)** Subcellular distribution of PAS2:GFP in BY-2 cells. Most of the interphasic cells showed PAS2:GFP accumulation outside the nucleus **(H)** but could also be detected strongly accumulated inside the nucleus in a minority of cells **(I)**. PAS2:GFP fusion was found to embrace chromosomes during the different phases of mitosis as shown for metaphase **(J)**, anaphase **(K)**, and telophase **(L)**. For each panel, the GFP signal, the DRAQ5 nuclear staining, and merged image are shown in the top, middle, and bottom panels, respectively.
Bars = 1 mm (**[A]**, **[B]**, and **[D]**), 25 μm **(E)**, 5 μm (**[F]** and **[G]**), and 30 μM (**[H]** to **[L]**).

Subcellular distribution of PAS2 was also investigated in stable transgenic BY-2 cell lines producing the PAS2:GFP fusion protein. As observed in *Arabidopsis* seedlings, PAS2:GFP fusion protein was mainly found outside the nucleus with a strong accumulation in the perinuclear region (Figure 5H). Nonetheless, a regular, but limited, occurrence of nucleus-localized GFP signal could be found in BY-2 cells, similar to that observed in *Arabidopsis* (Figure 5I). During mitosis, PAS2:GFP was tightly associated with the chromosomes capping their distal side during metaphase (Figure 5J), anaphase (Figure 5K), and late telophase (Figure 5L).

DISCUSSION

PAS2 is a member of the PTPL family that is conserved among eukaryotes. The PTPL family most probably fulfills important functions because PAS2 is not only essential in *Arabidopsis* but also in yeast and mammals (Bellec et al., 2002; Pelé et al., 2005). The function of PAS2 is also conserved because the *Arabidopsis PAS2* gene is able to complement yeast lethality (Bellec et al., 2002). The fact that PAS2 has an essential function that has been maintained from yeast to plants suggests that PAS2 is involved in a highly evolutionarily conserved cellular process.

Here, we provide evidences that PAS2, as the first plant member of the PTPL family, is involved in the regulation of cell division. PAS2 has been found to bind specifically to Tyr phosphorylated CDKA;1, suggesting that it has lost the PTP catalytic activity but not its substrate binding affinity. In animals, the dual dephosphorylation of Thr-14 and Tyr-15 of CDK by CDC25 is responsible for CDK2 activation. We demonstrated that PAS2 is also able to bind to WEE1-phosphorylated CDK2, confirming not only the biochemical conservation of PAS2 function through evolution from yeast to human but also the importance of the regulatory Tyr-15 for PAS2 interaction. Nonetheless, Thr-14 phosphorylation is also probably involved in PAS2 CDK interaction, which is consistent with the conservation of Thr-14 among plant CDKAs and its known regulatory role in CDK activity. The combined mutation of Thr-14A and Tyr-15F in *Arabidopsis* CDKA;1 led to constitutively active CDKA;1, as demonstrated in yeast by premature cell division (Hemerly et al., 1995). Overexpression of this mutant form of CDKA;1 in *Arabidopsis* provokes a mild phenotype with loss of apical dominance (Hemerly et al., 1995), which is reminiscent of cytokinin-overproducing plants (Howell et al., 2003). Interestingly, cytokinins promote cell division by upregulating a CDC25-like activity in *N. plumbaginifolia* cells, suggesting that the positive effect of cytokinins on cell division is caused by CDKA;1 dephosphorylation and activation (Zhang et al., 1996, 2005). In the *pas2* mutant, the dephosphorylation and activation of CDKA is thus consistent with the increase in cell proliferation induced by cytokinin application (Faure et al., 1998). PAS2 and cytokinins have antagonistic effects on CDC25-like activity, the activation of CDKA;1, and consequently on cell division.

Our work suggests also that Tyr phosphorylation is important for regulating cell proliferation. Phosphorylated Tyr is not a common posttranslational modification in plants compared with animals. Nonetheless, several PTPs and kinases have been described in *Arabidopsis* (Gupta et al., 1998; Xu et al., 1998; Fordham-Skelton et al., 1999, 2002; Ulm et al., 2001, 2002). Tyr phosphorylation has also been involved in several physiological responses, such as bending or phytohormone-stimulated cell proliferation (Kameyama et al., 2000; Huang et al., 2003). We show here that the level of CDKA;1 Tyr phosphorylation is controlled by PAS2. The mode of action of PAS2 is reminiscent of PTP inhibitors or antiphosphatases, such as STYX and Sbf1, which are inactive dsPTPs involved in suppression of cell transformation and in cell differentiation (Wishart et al., 1995; Cui et al., 1998). First, PAS2 associates only with phosphorylated CDKA;1. Then, the absence of PAS2 leads to a decrease in Tyr phosphorylation levels of CDKA;1 probably by directly inhibiting CDKA;1 activation by a CDC25-like activity. The previously identified plant CDC25, *Arabidopsis* CDC25;1, might not be a bona fide

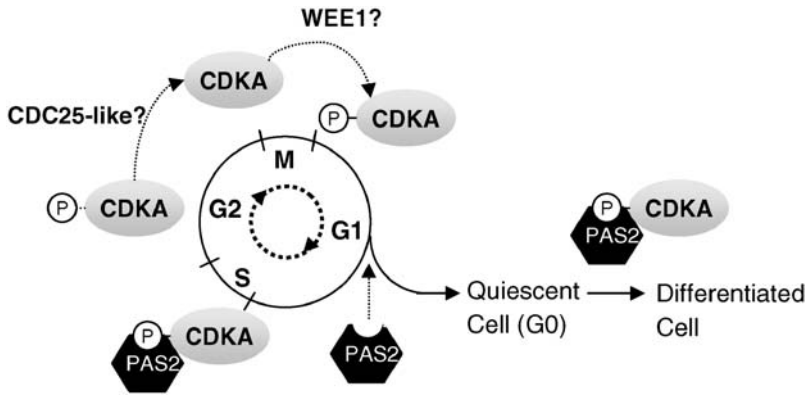

Figure 6. Model of PAS2 Action as an Antiphosphatase.

During the cell cycle, CDKA;1 activity is regulated by inhibitory phosphorylation (P). WEE1 is the kinase that most probably inactivates CDKA;1 after mitosis; thereafter, CDKA;1 is progressively dephosphorylated to the onset of mitosis by phosphatase(s). Cells could continue cycling or could be committed to the quiescent cycling state (G0) or a differentiated state. By maintaining CDKA;1 in a hyperphosphorylated inactive form, PAS2 could directly inhibit cell division and induce the cell to differentiate. Alternatively, once cells have exited from the cell cycle, PAS2 would be required to hold the cells committed to their differentiation state

CDC25 because it is probably involved in arsenate reduction (Bleeker et al., 2006). An attractive model would be that plants have nonspecific phosphatases that dephosphorylate CDKA unless it is protected by proteins such as PAS2. In such a case, PAS2 overexpression would antagonize several phosphatase(s) involved in different phases of the cell cycle leading to apparently contradictory results like the acceleration of G2 exit and delay in early mitosis.

Thus, we propose the following model where the cell cycle progression and the entry into mitosis is controlled by CDKA;1 phosphorylation (Figure 6). Premature mitosis is prevented by the WEE1 kinase that phosphorylates CDKA;1 after mitosis. PAS2 would then maintain CDKA;1 in a phosphorylated and inactive state, preventing the premature action of phosphatases. The next cell division would then be initiated upon the action of a CDC25-like activity by signals such as cytokinins. The dissociation of PAS2 from CDKA;1 might have two explanations: an increase of the CDC25-like phosphatase activity at the onset of mitosis sufficient to dephosphorylate CDKA and thus release PAS2 or an active mechanism removing PAS2 from the CDKA complex and allowing access to the CDC25-like phosphatase. A precise analysis of CDC25-like activity during the cell cycle would discriminate between the two mechanisms.

According to this model, PAS2 action is expected to colocalize intracellularly with its target. Contrary to *Arabidopsis* CDC25;1, the subcellular localization of CDKs has been described in several reports. Immunolocalization experiments showed that CDKs are mainly located in the nucleus in interphase and up to early prophase, and during mitosis, they are associated with chromatin, the preprophase band, the mitotic spindle, and the phragmoplast (Colasanti et al., 1993; Bögre et al., 1997; Stals et al., 1997). CDKA was also seen in the cytosol of *Medicago sativa* cells during the G2 phase (Bögre et al., 1997). These different localizations were confirmed by dynamic analysis of a *Medicago* CDKA:GFP fusion protein (Weingartner et al., 2001). The presence of PAS2 closely associated with mitotic chromosomes is consistent with its effect during cell division and supports the hypothesis of an interaction between PAS2 and CDKA;1 at G2/M or early mitosis to prevent premature cytokinesis. In actively dividing cells, such as in the root meristem or in BY-2 cells, PAS2 is principally excluded from the nucleus, and it remains to be determined whether PAS2 interacts with the cytosolic fraction of phosphorylated CDKA;1 or if the low amount of PAS2 remaining in the nucleus throughout the cell cycle could be sufficient for CDKA regulation. Nonetheless, occasional nuclear accumulation of PAS2 in interphasic BY-2 cells could be interpreted either as a premitotic mark or as a sign of resting or quiescent cells. The latter interpretation is supported by the fact that PAS2:GFP is clearly not excluded anymore from the nucleus in differentiated *Arabidopsis* root cells, probably downregulating CDKA;1 and preventing the cells from reentering mitosis (Figure 6). Alternatively, PAS2 might fulfill different functions in the nucleus and in the cytosol. In both cellular compartments, PAS2 might act as an antiphosphatase on CDKA but toward different phosphatases: one involved in the nucleus controlling the G2/M transition and another involved in the cytosol in the maintainance of a quiescent or differentiated cellular state. While in the absence of PAS2, cells are more prone or competent for cell division, and *PAS2* overexpression, as expected, inhibits the growth of young leaves and, in the strongest case, the development of leaf primordia. The fact that cotyledons of transgenic PAS2:GFP have a phenotype similar to early onset of senescence could be a sign of accelerated cell differentiation. Thus, it is likely that PAS2 has a distinct function in dividing and differentiated cells (i.e., in dividing cells, PAS2 would interact transiently or at low levels with CDKA;1), whereas in differentiated cells, the interaction would be increased and stabilized. The role of PAS2 at the interface of cell division and differentiation is also illustrated by its expression pattern during early embryo development (Casson et al., 2005). *PAS2* is one of the most differentially expressed genes in the apical zone of the globular embryo, and its expression precedes and marks the onset of cotyledon initiation. This developmental stage is characterized by high proliferative activity of cells and by the main differentiation step, leading from radial to bilateral symmetry during embryo development.

In conclusion, PAS2 might represent a clue to understand how plants maintain a high cellular plasticity with coordinated cell differentiation. The conservation of sequences and functions in the PTPL family would also suggest that PAS2 might represent a general regulatory mechanism involved in the control of cell proliferation. Deciphering the precise role of PAS2 during cell differentiation and in particular how its intracellular localization is regulated will be necessary to fully understand the function of its inactive phosphatase.

METHODS

Materials

The protein fusions PAS2:GFP and PAS2:His were constructed by cloning the full-length cDNA of *PAS2* (666 bp) into the Gateway-modified vector pK7WG2D (Karimi et al., 2005) and into the expression vector pIVEX2.4 (Roche Diagnostics), respectively. The *CDKA;1* gene was cloned into an MBP tag-containing pMALC2X vector and resulted in the MBP:*CDKA;1* expression construct.

The peptide CMLGQRKRALSKSKRE-amide from the PAS2 sequence was synthesized and used as antigen in rabbits to produce the antiserum (anti-PAS2) (Biogenes). The anti-PSTAIRE (anti-CDKA) is a mouse IgG monoclonal antibody (Sigma-Aldrich; reference P7962). For detection of Tyr phosphorylated CDKA, a mouse IgG polyclonal antibody anti-PTYR was used (Santa Cruz Biotechnology; reference Sc7020). Phosphorylated Tyr-15 was detected by a rabbit polyclonal antibody (Cell Signaling Technology; reference 9111).

Protein–Protein Interaction and Phosphorylation in Vitro

Production of recombinant CDKA;1 was performed after induction of BL21(DE3)pLysE cells carrying the MBP:CDKA;1 construct by 2 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h at 37°C. After one wash in lysis buffer (40 mM Tris, pH 8, 100 mM NaCl, 50 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM vanadate, and protease inhibitor), cells were resuspended in 1 mL lysis buffer with 4 mg/mL lysozyme and incubated 15 min at room temperature. The cells were sonicated on ice twice (15 pulses of 1 s) and centrifuged (25 min at 4°C; 6000*g*). The supernatant was incubated for 30 min at room temperature with 50 μL amylose matrix (50%) and washed twice with binding buffer (10 mM Tris, pH 7.5, 75 mM NaCl, 1 mM DTT, 1 mM vanadate, 1 mM EDTA, and protease inhibitor). In vitro Tyr phosphorylation of MBP:CDKA;1 with the Src kinase (Upstate

Biotechnology; reference 14-117) was performed by incubation of the MBP:CDKA;1 maltose matrix with 25 units of Src and ATP (or [γ-^{32}P]ATP) and detected with the BAS 1500 imaging analyzer (Fuji) in phosphorylation buffer (100 mM Tris, pH 7.5, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 1 mM vanadate, and protease inhibitor) for 1 h at 30°C. The beads were washed three times with binding buffer, and MBP:CDKA;1 was eluted with 15 mM of maltose. The PAS2:His protein was produced by the RTS100 *Escherichia coli* kit according to the manufacturer's instructions (Roche Diagnostics). PAS2:His was purified on nickel beads blocked with 3% BSA, 0.1% Tween 20, and 1% Triton X-100 in binding buffer for 30 min at room temperature and washed with 1% Triton X-100 in PBS. An RTS100 reaction containing PAS2:His was incubated with the nickel Sepharose matrix for 30 min and washed three times with binding buffer.

MBP:CDKA;1 phosphorylated or not by Src kinase was incubated with the PAS2:His matrix for 30 min at room temperature, washed with binding buffer, and finally eluted by boiling the matrix in Laemmli buffer. The presence of MBP:CDKA;1 phosphorylated or not was revealed by protein gel blotting with an anti-PSTAIRE antibody.

GST:CDK2 expression and phosphorylation by WEE1 in *E. coli* extracts was performed as previously described (Welburn and Endicott, 2004). After purification of CDK2 or CDK2-WEE1 on glutathione beads, proteins were eluted using reduced glutathione and incubated 2 h at 4°C with nickel columns containing either PAS:His or His tag alone. The nickel columns were blocked as described before, and after several washes, the proteins were eluted by boiling the matrix in Laemmli buffer. The presence of CDK2 was analyzed by protein gel blotting using the PSTAIRE antibody. The phosphorylation status of CDK2 or CDK2-WEE1 was revealed by protein gel blotting with the antiphosphorylated Tyr-15. The competition experiments were performed by washing CDK2-WEE1 bound to PAS:HIS columns with 1 mM of the following peptides: EKVEKIGEGpTYGVVYK (pThr-14), EKVEKIGEGTYGVVYK (control peptide), and EKVEKIGEGTpYGVVYK (pTyr-15). The presence of CDK2-WEE1 was analyzed by protein gel blotting using the PSTAIRE antibody as described before.

Whole-Plant Extracts and Pull-Down Assays

Approximately 3 g (fresh weight) of cells of *Arabidopsis thaliana* were ground in liquid nitrogen, and ~7 mg/mL of proteins were extracted in 5 mL of extraction buffer (25 mM Tris, pH 7.6, 15 mM $MgCl_2$, 15 mM EGTA, 85 mM NaCl, 15 mM pNO_2PhePO_4, 60 mM B-glycerophosphate, 1 mM DTT, 0.1% Nonidet P-40, 1 mM vanadate, 1 mM NaF, and protease inhibitor). For pull-down assays, 30 μg of PAS2:His recombinant protein produced from a RTS500 reaction (Roche Diagnostics) was adsorbed on nickel Sepharose as described above. Proteins from *Arabidopsis* were incubated with an empty sepharose or with PAS2:His nickel Sepharose overnight at 4°C. After three washes with binding buffer, the proteins were eluted by boiling in Laemmli buffer. The Tyr-phosphorylated form of CDKA;1 was detected using the anti-PTYR antibody.

For CDKA;1 and PAS2 in vivo interaction, *Arabidopsis* cells or seedlings were ground in liquid nitrogen with extraction buffer, and ~600 μg protein extract was incubated with 50 μL of p10^{CKS1At} beads prepared as described by Brizuela et al. (1987) and Landrieu et al. (1999) for 2 h at 4°C. The beads were washed twice with bead buffer (50 mM Tris, pH 7.5, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM vanadate, and protease inhibitor). Protein extracts bound to p10^{CKS1At} beads were analyzed by protein gel blotting with the anti-PSTAIRE and anti-PAS2 antibodies.

Kinase Activity of CDKA;1

CDKA;1 from *Arabidopsis* seedlings was purified as described above. The p10CKS1At beads were washed twice with bead buffer and once with kinase buffer (50 mM Tris, pH 7.8, 15 mM $MgCl_2$, 5 mM EGTA, and 1 mM DTT). In vitro histone H1 kinase was assayed as described by Magyar et al. (1997). The samples were analyzed by 12% SDS-PAGE, stained with Coomassie Brilliant Blue R 250, and analyzed with BAS1500 to detect histone H1 phosphorylation. Samples were normalized according to PSTAIRE labeling. For competition assays between PAS2 and *Arabidopsis* CDC25, we first purified CDKA;1 from *Arabidopsis* cells using p10CKS1At as described above. The beads were washed twice with bead buffer and once with kinase buffer. The histone H1 kinase activity was assayed in vitro as described above.

RT-PCR Methods

RNA were extracted and treated with DNase according to the RNeasy plant kit (Qiagen). Reverse transcriptions were performed from total DNase-treated RNA with superscript II enzyme (Qiagen) according to standard protocols. PCRs were performed with primers for *Arabidopsis PAS2* (5′-CCATGAAGAATCTCGAGAACG-3′ and 5′-TCTATGACGCCATTGAGAAGC-3′) and *Nicotiana sylvestris atp2* as control (5′-GTGAAGAGGCGCGTGAAG-3′ and 5′-GTCTAATTTCCCGATCGTTAGGA-3′).

Cell Analysis

Nicotiana tabacum BY-2 cells were grown in Murashige and Skoog medium in a growth chamber under constant darkness and 25°C (Joubès et al., 2004). For Cre/Lox induction, a 3-d-old culture was agitated for 2 h at 37°C and cooled down for 5 h at 25°C. Finally, 10 μM Dex (Sigma-Aldrich) was added. For synchronization, 5 mg/L aphidicolin was added 24 h after HS, and cells were blocked for 24 h. Cells were washed extensively with fresh medium, resuspended in conditioned medium subculture, and sampled at different times. Cells were washed, fixed in 0.1 M citric acid and 1% Triton X-100, and chopped with a razor blade. After filtration over a 30-μm mesh, the nuclei were stained with 0.1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI) and analyzed with a UV flow cytometer (EPS Elite; Beckman-Coulter). The mitotic index was monitored on DAPI-stained cells. Confocal microscopy was performed on an inverted TCS-SP2-AOBS spectral confocal laser scanning microscope (Leica Microsystems) with an HCX PL APO ×63/1.2w long-working-distance (220 μm) water immersion objective (Leica). Samples were excited with a 488-nm argon laser with an emission band of 500 to 510 nm for GFP detection and with a 633-nm HeNe laser with an emission band of 684 to 735 nm for the detection of the vital DNA marker DRAQ5 (100 μM).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AY047708 (PAS2), DQ158862 (CDKA;1), and NM120425 (*Arabidopsis* CDC25).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Complementation of the *pas2.1* Mutant Phenotype by the *35S-PAS2* Construct.

Supplemental Figure 2. Nuclear Accumulation of PAS2:GFP in the Root Hair Cell.

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